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Biodegradation of Glyceryl Trinitrate by *Penicillium corylophilum* Dierckx

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Penicillium corylophilum **Dierckx, isolated from a contaminated water wet, double-base propellant, was able to completely degrade glyceryl trinitrate (GTN) in a buffered medium (pH 7.0) containing glucose and ammonium nitrate. In the presence of 12 mg of initial fungal inoculum, GTN (48.5 to 61.6 μmol) was quantitatively transformed in a stepwise process to glyceryl dinitrate (GDN) and glyceryl mononitrate (GMN) within 48 h followed by a decrease in the GDN content with a concomitant increase in the GMN level. GDN was totally transformed to GMN within 168 h, and the complete degradation of GMN was achieved within 336 h. The presence of glucose and ammonium nitrate in the growth medium was essential for completion of the degradation of GTN and its metabolites. Complete degradation of GTN by a fungal culture has not been previously reported in the literature.**

Glyceryl trinitrate (GTN) is one of the major ingredients in double-base propellant compositions (17), which are used in gun and rocket formulations and for the casing of intercontinental ballistic missile motors. The manufacture of GTN, the generation of scrap propellant, and the need to demilitarize the munitions produce large amounts of waste materials containing GTN. Demilitarization of these items has become a significant environmental concern in recent years. Current disposal techniques such as open-air burning and incineration, which produce hazardous waste, coupled with the mammalian toxicity and genotoxicity of GTN (5, 9, 17), point out the need for more environmentally friendly disposal methods. Although several chemical methods for the disposal of GTN have been reported, they are not desirable because of incomplete degradation, large consumption of chemicals, evolution of toxic or offensive gases, and their relatively high cost (1). However, microbial degradation processes hold the potential for more environmentally acceptable alternatives to the current disposal methods (open-air burning, open-air detonation, and incineration).

The aerobic and anaerobic microbial degradation of GTN has been previously investigated (2–4, 11, 19, 21, 22). None of these investigations reported the complete denitration of GTN. Ducrocq et al. (3, 4) showed that certain yeast and mycelial fungi can convert GTN to a mixture of glyceryl dinitrates (GDNs) and glyceryl mononitrates (GMNs). Wendt et al. (19), in their pioneering studies, proposed that the bacterial metabolism of GTN occurs via sequential denitration to GDN and GMN and speculated that the GMN was further denitrated to form glycerol. However, the degradation was not complete, as GDN and GMN were still present in the spent medium (19). Recently, Meng et al. (10) reported that GTN can be completely denitrated during a long-term incubation with cell extracts of either *Bacillus thuringiensis* plus *Bacillus cereus* or *Enterobacter agglomerans*. Although the method of Meng et al. appears to effectively degrade GTN, it must be pointed out that *B. cereus* and *E. agglomerans* are mammalian pathogens whereas *B. thuringiensis* is an insect pathogen (6, 12, 16). Further, the authors pointed out that this method is suitable only for the small-scale degradation of GTN (10).

Previously, we reported the isolation of a fungal culture, which was identified as *Penicillium corylophilum* Dierckx, from a water wet, contaminated double-base propellant (14). Of all the ingredients in double-base propellants, only GTN has appreciable aqueous solubility. It is likely that the organism was utilizing GTN for growth. We previously demonstrated its ability to degrade nitrocellulose (14). To use this culture for the disposal of double-base propellant formulations, it was necessary to study its ability to degrade other major ingredients present in the composition. Hence, we carried out a systematic study of the biodegradation of GTN by *P. corylophilum.*

MATERIALS AND METHODS

Organism. *P. corylophilum* was isolated from a contaminated, water wet double-base propellant which contained essentially nitrocellulose (~84%), GTN (~11%), 2,4-dinitrotoluene (~0.1%), diphenylamine (~1%), and di-*n*-butyl phthalate (~4%) (14).

Maintenance of culture. *P. corylophilum* was maintained at 4°C on Sabouraud maltose agar with monthly transfers.

GTN and its derivatives. GTN was obtained as a 10% solution in propylene glycol from Zeneca (Wilmington, Del.). 1,2-GDN, 1,3-GDN, 1-GMN, and 2-GMN were obtained as 100-μg ml^{-1} solutions in acetonitrile from Radian Corp. (Austin, Tex.).

Growth medium. Mineral salts medium used in this study contained the following (in grams per liter): KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1; and $CaCl_2 \cdot 2H_2O$, 0.1. This was made up with 100 μl of trace metal salt solution, which contained the following: $C_6H_8O_7$ (citric acid), 5.0%; $ZnSO_4 \cdot 7H_2O$, 5.0%; $CuSO_4 \cdot 5H_2O$, 0.25%; $MnSO_4 \cdot H_2O$, 0.25%; H_3PO_4, 0.05%; $Na_2MoO_4 \cdot 2H_2O$, 0.05%; and $CoCl_2 \cdot 6H_2O$, 2.0%.

Inoculum preparation. Mycelial mats were pregrown in mineral salts medium containing xylan (0.5%) as a carbon source and NH_4NO_3 (0.1%) as a nitrogen source. Fungi were grown at 28°C in a gyratory shaker (~150 rpm) for 96 h and harvested by centrifugation (3,014 × *g* at 4°C for 10 min). The harvested fungal mycelia were washed thoroughly under aseptic conditions with the mineral salts medium lacking carbon and nitrogen sources. The washed fungal mycelia were homogenized with a Virtishear explosion-proof pilot homogenizer operated at half of the maximum output for 2 min at 4°C with 15-s interruptions.

Determination of the inoculum biomass weight. A known volume of homogenized mycelia of *P. corylophilum*, used for inoculation, was passed through a preweighed filter paper, washed with distilled water, and dried at 105°C until a constant weight was reached to determine the inoculum weight (7).

Culture conditions. Equal volumes of homogenized mycelia (~12 mg [dry weight]) were transferred to the growth medium (50-ml final volume in individ-

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ual 250-ml Erlenmeyer flasks) containing GTN (48.5 to 61.6 μmol [see Tables 1 and 2]), additional glucose (8%), and ammonium nitrate (0.4%) buffered at pH 7.0 with KH_2PO_4 and Na_2HPO_4 (130 mM). The inoculated flasks were incubated for various time intervals in a gyratory shaker (~150 rpm at 28°C). Four types of controls were run in parallel to the treatment; one of them lacked GTN but contained the culture, one contained NaN_3 (1 mM) and $HgCl_2$ (1 mM) along with the culture, one contained only GTN, and one was run in the presence of propylene glycol and *P. corylophilum* (but in the absence of glucose and GTN). This was done to determine if this compound could provide sufficient carbon to support fungal growth since it is the solvent for the GTN stock solution. No fungal growth was observed at the level of propylene glycol introduced into each culture flask.

Determination of GTN and its transformation products adsorbed to the biomass. The biomass was filtered off from the growth medium and washed thoroughly with appropriate volumes of water. It was then suspended in water and homogenized with a Virtishear explosion-proof pilot homogenizer operated at half of the maximum output for 2 min at 4°C with 15-s interruptions. The homogenate was centrifuged (8,000 × *g*) at 28°C for 15 min. The pellet was further extracted with methanol and centrifuged (8,000 × *g*) at 28°C for 15 min. The GTN, GDN, and GMN contents in the washings, aqueous homogenate supernatant, and methanol extract of the pellet were then determined by high-pressure liquid chromatography (HPLC) as described below.

Analyses of GTN and its transformation products. The concentrations of GTN, GDN, and GMN in the growth medium and in the other fractions (filtered with a 0.45-μm-pore-size syringe filter) were determined by HPLC (model 501 chromatograph; Waters, Milford, Mass.) equipped with a Partisil 10 ODS-3 column (4.6 by 250 mm; Whatman). Two sets of HPLC elution conditions were used for the separation of GTN and its degradation products. Method 1 consists of a mobile phase with 60% acetonitrile and 40% water. Method 2 consists of a mobile phase with 5% acetonitrile and 95% water. The GTN could not be separated by method 2, and GMN could not be separated by method 1. Hence, method 1 was used at the 0- to 48-h time points for the GTN and GDN determinations and method 2 was used at the 0- to 336-h time points for GDN and GMN determinations. Data presented in Tables 1 and 2 were generated by method 1 at the 0- to 24-h time points and by method 2 at the 48- to 336-h time points. In all cases, the flow rate was 1.0 ml min^{-1} and the detection was carried out at 204 nm. The retention times were 6.95, 5.40, and 3.20 min for GTN, GDN, and GMN, respectively, in method 1. In method 2, the retention times were 34.24, 27.76, 7.84, and 7.24 min for 1,2-GDN, 1,3-GDN, 2-GMN, and 1-GMN, respectively.

RESULTS AND DISCUSSION

The initial experiment directed towards the degradation of GTN by *P. corylophilum* (biomass dry weight, 4.0 mg) with 0.6% glucose but without an additional nitrogen source resulted in 51% of the GTN (initial content, 11 μmol) being transformed to a mixture of GDN and GMN by 166 h with an increase in the biomass weight to 19.3 mg. This result is consistent with those of Ducrocq et al. (3, 4), who showed that *Phanerochaete chrysosporium* and *Geotrichum candidum* can convert GTN to a mixture of GDN and GMN in the presence of a carbon cosubstrate. Attempts were made to improve the degradation by adding 0.08% ammonium nitrate as a supplemental nitrogen source to the growth medium (18). By 166 h, about 64% of the GTN was transformed to a mixture of GDN and GMN.

Efforts were made to identify the culture conditions required to achieve complete degradation of GTN, GDN, and GMN. Based on a series of preliminary studies, it was found that the inoculum size, amounts of additional carbon and nitrogen sources, and pH were major factors that effected the extent and rate of degradation. The preliminary experiments were carried out at 0, 0.1, 0.2, 0.3, 0.4, and 0.5% ammonium nitrate along with glucose as a supplemental carbon source. In the absence of ammonium nitrate, the GTN was converted to a mixture of GDN and GMN. Upon addition of lower levels of ammonium nitrate, the GDN was converted to GMN. It appears that the amount of nitrogen available from GMN was not sufficient to support the fungal growth. Complete disappearance of GTN (48.5 to 61.6 μmol) was obtained with 12 mg (dry weight) of *P. corylophilum* in 50 ml of growth medium containing 8% glucose and 0.4% ammonium nitrate buffered at pH 7.0 with 130 mM phosphate buffer.

TABLE 1. Time course of GTN degradation by *P. corylophilum* Dierckx[a]

Time (h)	Amt (μmol) of:					Total amt (μmol)
	GTN	GDN		GMN		
		1,2-GDN	1,3-GDN	1-GMN	2-GMN	
0[b]	48.5	0	0	0	0	48.5
19[b]	37.5	3.0	1.5	0	0	42.0
48[c]	0	16.0	10.0	1.0	1.5	28.5
92[c]	0	3.0	5.5	12.0	6.0	26.5
164[c]	0	0	0	18.0	7.5	25.5
260[c]	0	0	0	0	7.0	7.0
284[c]	0	0	0	0	2.0	2.0
308[c]	0	0	0	0	0	0

[a] Initial GTN content, 48.5 μmol. The experiment was conducted in a single flask, and 15 μl was used at each time point for HPLC analysis.
[b] The mobile phase containing 60% acetonitrile and 40% water was used for the HPLC separation of GTN and its products.
[c] The mobile phase containing 5% acetonitrile and 95% water was used.

A systematic time course study was then conducted with the reagents in a single flask. In this study, it was observed that the GTN was absent from the medium after 48 h and only GDN (1,2-GDN and 1,3-GDN) and GMN (1-GMN and 2-GMN) remained (Table 1). The GDN content decreased with time and was zero at 164 h with only GMN remaining in the medium. The degradation of GMN was much slower and was completed by 308 h. A significant loss in the mass balance of GTN was observed in the medium before the formation of GMN. This is not consistent with the stepwise degradation mechanism proposed by earlier investigators (8, 13, 15, 19, 20). Because of the apparent lack of mass balance and the presence of a large quantity of biomass in the growth medium (Table 2), we studied the possibility of the adsorption of GTN and its degradation products by the biomass. This was done in another set of experiments in individual flasks by filtering off the biomass from the medium and thoroughly washing it with water. The biomass was then homogenized and centrifuged, and the pellet was extracted with methanol. The GTN, GDN, and GMN contents in the filtrate, washings, aqueous supernatant, and methanol were determined (Table 3). A significant amount of GTN and its degradation products were detected in the washings and in the medium. However, no GTN or its degradation products were detected in the aqueous homogenate supernatant or in the methanol extract of the pellet.

A gradual decrease in the amount of adsorbed material was observed up to the 72-h time point (Table 3). At 168 h, no adsorbed GTN or degradation products were detected. By adding up the number of micromoles of GTN and its degra-

TABLE 2. Growth pattern of *P. corylophilum* Dierckx in the presence of GTN

Time (h)	Amt (mg) of biomass[a]
0	12
19	20
24	52
48	192
72	339
168	444
336	839

[a] The growth pattern of *P. corylophilum* in the presence of 61.5 μmol of GTN, 8% glucose, and 0.4% ammonium nitrate.

TABLE 3. Time course of GTN degradation by *P. corylophilum* Dierckx and extraction of GTN adsorbed to the biomass

Time (h)	Initial amt (μmol) of GTN	Amt (μmol), in growth medium, of:					Total amt (μmol) in medium	Amt (μmol) of GTN+GDN+GMN adsorbed to biomass	Total amt (μmol) of GTN and degradation products[c]
		GTN	1,2-GDN	1,3-GDN	1-GMN	2-GMN			
0[a]	59.1	59.1	0	0	0	0	59.1	0	59.1
19[a]	58.0	39.7	7.6	0	0	0	47.3	10.5	57.8
24[a]	58.9	34.0	7.0	4.7	0	0	45.7	9.6	55.4
48[b]	59.6	0	14.6	8.8	1.8	1.5	26.7	8.0	34.7
72[b]	57.0	0	4.1	3.4	9.1	6.1	22.7	7.8	30.5
168[b]	61.6	0	0	0	16.6	10.3	26.9	0	26.9
336[b]	60.9	0	0	0	0	0	0	0	0

[a] The mobile phase containing 60% acetonitrile and 40% water was used for the HPLC separation of GTN and its products.
[b] The mobile phase containing 5% acetonitrile and 95% water was used.
[c] GTN and its degradation products remaining in the growth medium and adsorbed to the biomass. About 99.5% of the added GTN was accounted for in the killed control ($HgCl_2$ and NaN_3).

dation products in the medium and those adsorbed on the biomass, we could account for almost all GTN up to the point where GMN formed. From 48 h, GMN appeared along with GDN. At the 336-h growth period, the GMN was completely degraded by *P. corylophilum* as evidenced by its absence from the medium as well as from the biomass fractions. These results support the stepwise denitration mechanism. Further studies including identification, purification, and characterization of the enzymes involved in the GTN degradation are currently under way in our laboratory.

Overall, GTN can be completely degraded by using a nonpathogenic fungal culture, *P. corylophilum*. To the best of our knowledge, the complete fungal denitration of GTN has not been previously reported. In the present investigation, a significant increase in the biomass content was observed during the GTN denitration process (Table 2). The biomass data indicates that the GTN, GDN, and GMN removal occurred during fungal growth. Our results show that in the absence of ammonium nitrate, the GTN was converted to a mixture of GDN and GMN. In the presence of low levels of ammonium nitrate, significant amounts of GMN were still present in the culture medium. The data indicates that at least 0.4% ammonium nitrate is necessary for the complete denitration of GMN. It appears that the presence of ammonium nitrate is necessary for the production of the enzymatic system responsible for the denitration of GMN. It is likely that the nitrogen released from the GDN-to-GMN conversion was insufficient to support fungal growth and thus to produce the enzyme system required for GMN denitration. Once the enzymatic mechanism for GMN degradation is elucidated, it may be possible to select a less expensive nitrogen source for the practical implementation of this technology.

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